

BASIC PROTOCOLS
FOR LEVEL A LABORATORIES

FOR THE PRESUMPTIVE
IDENTIFICATION OF

Yersinia pestis

CDC
Centers for Disease Control
and Prevention

ASM
American Society for Microbiology

APHL
Association of Public Health Laboratories

Credits: *Yersinia pestis*

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I. General information

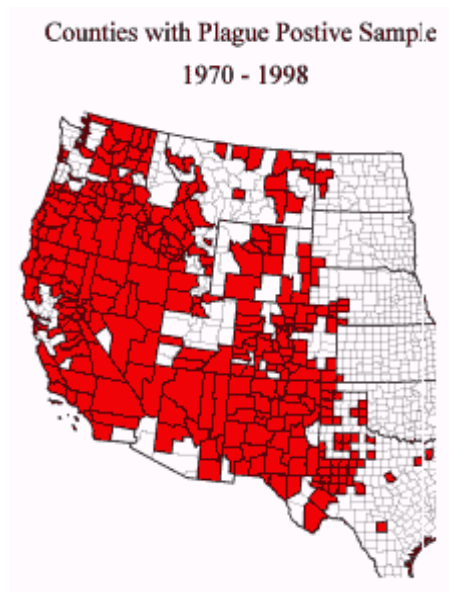
A. Description of organism

Yersinia pestis is a nonmotile, slow-growing, facultative organism classified in the family *Enterobacteriaceae*. It appears as plump, gram-negative coccobacilli that are seen mostly as single cells or pairs, and which may exhibit bipolar staining from a direct specimen.

B. History

Y. pestis, the causative agent of plague, has a protracted history, being described in epidemics and pandemics since biblical times. In the Middle Ages, it was estimated to have killed up to 40% of the European population (Butler, 2000). In more recent history, pandemic plague began in China in the 1860s. It spread to Hong Kong by the 1890s and subsequently was spread by ship rats to the Americas, Africa, and other parts of Asia (Perry, 1997). As recently as the beginning of the 20th Century, India suffered more than 10 million deaths from plague, and in the 1960s and 1970s, Vietnam was engrossed in a plague epidemic (Butler, 1983). Numerous references in art, literature, and monuments attest to the horrors and devastation associated with the plague bacillus.

C. Geographic distribution in the United States



Plague is a zoonotic disease transmitted ordinarily from animals and their infected fleas. To date, plague has not been transmitted east of the Rocky Mountains.

D. Clinical presentation

Humans can acquire plague through the bite of infected fleas, direct contact with contaminated tissue, or inhalation (Gage, 1998). Clinically, plague may present in bubonic, septicemic, and pneumonic forms (Perry, 1997). Bubonic plague is characterized by sepsis that is accompanied by the sudden onset of fever, chills, weakness, headache, and the formation of painful buboes (swelling of regional lymph nodes of the groin, axilla, or neck). Septicemic plague is similar to bubonic plague, but lacks the swelling of the lymph nodes. Pneumonic plague, the most deadly form of the disease and the form that can be transmitted rapidly, presents as fever and lymphadenopathy with cough, chest pain, and often hemoptysis. Secondary pneumonia from hematogenous spread of the organisms can occur (secondary pneumonic plague). The organism can also occasionally be passed from human to human by close contact as in primary pneumonic plague (Campbell, 1998). Primary pneumonic plague would probably be the form that would be seen if *Y. pestis* were used in a bioterrorism event.

II. Procedures: *Yersinia pestis*

- A. General:** The procedures described below function to rule out *Yersinia pestis* using specimens or isolates.
- B. Precautions:** These procedures should be performed in microbiology laboratories that use Biological Safety Level-2 (BSL-2) practices; use of biological safety cabinet is recommended. Because of the infectious nature of this organism, the state public health laboratory/department should be consulted immediately if *Y. pestis* is suspected.

C. Specimen

- 1. Acceptable specimens:** Specimens of choice will be determined by the clinical presentation.
 - a. Lower respiratory tract (pneumonic): Bronchial wash or transtracheal aspirate (≥ 1 ml). Sputum may be examined but this is not advised because of contamination by normal throat flora.
 - b. Blood (septicemic): Collect appropriate blood volume and number of sets per established laboratory protocol. Note: In suspected cases of plague, an additional blood or broth culture (general nutrient broth) should be incubated at room temperature (22–28°C), temperature at which *Y. pestis* grows faster. Do not shake or rock the additional broth culture so that the characteristic growth formation of *Y. pestis* can be clearly visualized.
 - c. Aspirate of involved tissue (bubonic) or biopsied specimen: Liver, spleen, bone marrow, lung. Note: Aspirates may yield little material; therefore, a sterile saline flush may be needed to obtain an adequate amount of specimen. Syringe and needle of aspirated sample should be capped, secured by tape, and sent to the laboratory.
- 2. Specimen handling**
 - a. Respiratory/sputum: Transport specimens in sterile, screw-capped containers at room temperature. If it is known that material will be transported from 2–24 h after collection, then store container and transport at 2–8°C.
 - b. Blood: Transport samples directly to the laboratory at ambient temperature. Hold them at ambient temperature until they are placed onto the blood culture instrument or incubator. Do not refrigerate. Follow established laboratory protocol for processing blood cultures.
 - c. Tissue aspirate/biopsy specimen: Submit tissue or aspirate in a sterile container. For small samples, add 1–2 drops of sterile normal saline to keep the tissue moist. Transport the sample at room temperature for immediate processing. Keep the specimen chilled if processing of the specimen will be delayed.
 - d. Swabs: A swab of tissue is not recommended. However, if a swab specimen is taken, the swab should be reinserted into the transport package for transport.
- 3. Rejection criteria**
 - a. Use established laboratory criteria.

- b. Dried specimens should be referred to the state public health laboratory.
- c. Environmental/nonclinical samples and samples from announced events are not processed by Level A laboratories; submitter should contact the state public health laboratory directly.

D. Materials

1. Media

- a. General nutrient rich media: Sheep blood agar (SBA) and equivalent
- b. General nutrient rich broth: Brain heart infusion (BHI) and equivalent
- c. Selective agar: MacConkey (MAC) or eosin methylene blue (EMB) agar
- d. Blood culture, standard blood culture system

2. Reagents

- a. Gram stain reagents
- b. Wright-Giemsa or Wayson stain
- c. Oxidase reagents
- d. Catalase reagent (3% hydrogen peroxide)
- e. Urease test (e.g., Christensen agar, biochemical kit)

3. Equipment/supplies

- a. Microscope slides
- b. Heat source for fixing slides: Burner (gas, alcohol), heat block
- c. Staining rack for slides
- d. Microscope with high power and oil immersion objectives
- e. Bacteriologic loops, sterile
- f. Incubator: Ambient atmosphere, 28°C and 35–37°C

Disclaimer: Names of vendors or manufacturers are provided as examples of suitable product sources; inclusion does not imply endorsement by the Centers for Disease Control and Prevention, the Department of Health and Human Services, the Federal Bureau of Investigation, the American Society for Microbiology, the Association of Public Health Laboratories, or any other contributor.

- E. Quality control:** Perform quality control of media and reagents according to package inserts, NCCLS document M22-A2, and CLIA standards, using positive and negative controls appropriate for each media and reagent. Document all quality control results according to standard laboratory practices.

F. Stains and smears

1. Gram stain

- a. Procedure: Perform Gram stain procedure/quality control per standard laboratory protocol. Smears for staining may be prepared in order of likely positive results (i.e., cultures, bubo aspirates, tissue, blood, and sputum specimens).

- b. Characteristics: Direct microscopic examination of specimens and cultures by Gram stain can provide a rapid presumptive identification. Stained specimens containing *Y. pestis* often reveal plump, gram-negative rods, 1–2 μm X 0.5 μm , that are seen mostly as single cells or pairs and short chains in liquid media (Fig. A1). Note: Patients with pneumonic plague may be secondarily infected with *Streptococcus pneumoniae*. Both of these organisms may be visualized in the sputum smears. It is imperative to evaluate such smears for the presence of gram-negative rods around the leukocytes (not necessarily intracellularly).
- 2. Other stains**
- a. Presence of bipolar cells in these smears should trigger the suspicion of plague. The Wright stain often reveals the bipolar staining characteristics of *Y. pestis*, whereas the Gram stain may not. The Wright-Giemsa stains are the most reliable for accurately highlighting the bipolar staining characteristics of these gram-negative rods (Fig. A2).
 - b. Wayson stain, another polychromatic stain, can be used instead of Wright-Giemsa.
- 3. Additional work:** Another smear may be prepared for referral to the state public health laboratory.

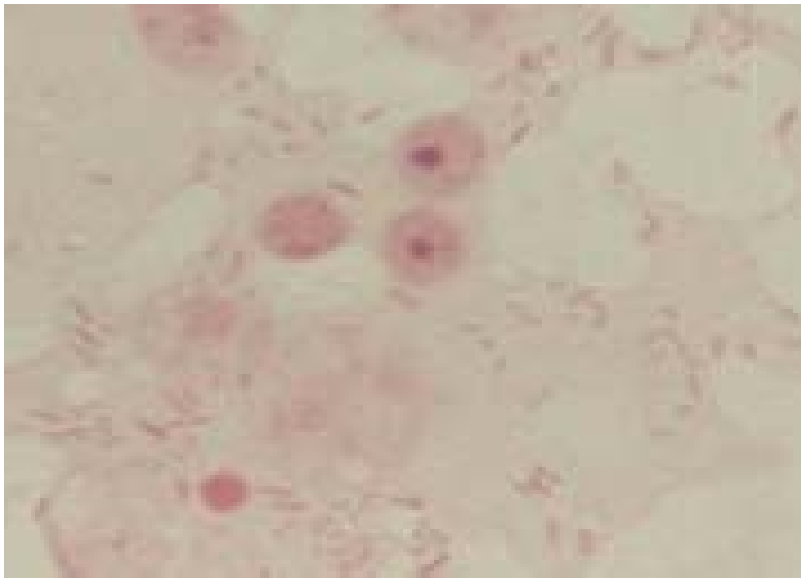


Figure A1. *Y. pestis* Gram stain, X1000

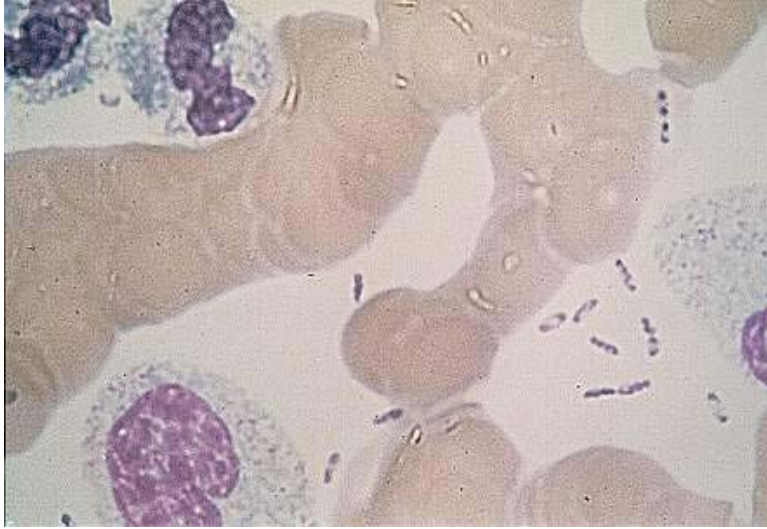


Figure A2. Giemsa stain of blood smear containing *Y. pestis* from a septicemic patient, X1000. Note the bipolar-staining cells.

G. Cultures

1. **Procedure:** Use established inoculation and plating procedure. For tissues, use established laboratory procedure to inoculate media (e.g., grind, touch-preparation, or by using a sterile wood stick). Then, tape plates shut in 2 places (or use alternative method) to prevent inadvertent opening.
2. **Incubation of cultures**
 - a. Temperature: 28°C (optimal); 35–37°C (grow more slowly).
 - b. Atmosphere: Ambient, use of 5% CO₂ is acceptable.
 - c. Length of incubation: Hold primary plates for 5 days. Plates should be held for up to 7 days if the patient has been treated with bacteriostatic antibiotic.
3. **Characteristics**
 - a. Agar plates: *Y. pestis* grows as gray-white, translucent colonies, usually too small to be seen as individual colonies at 24 h. After incubation for 48 h, colonies are about 1–2 mm in diameter, gray-white to slightly yellow, and opaque. Under 4X enlargement, after 48–72 h of incubation, colonies have a raised, irregular "fried egg" appearance, which becomes more prominent as the culture ages (Fig. A3a). Colonies also can be described as having a "hammered copper," shiny surface (Fig. A3b). There is little or no hemolysis of the sheep red blood cells. *Y. pestis* will grow as small, nonlactose fermenting colonies on MAC or EMB agar.
 - b. Broth tubes: *Y. pestis* grows in clumps that are typically described as "flocculant" or "stalactite" in appearance when the broth culture is not shaken or mixed. At 24 h, the growth is seen as clumps that hang along the side of the tube. After 24 h the growth settles to the bottom of the tube described as "cotton fluff."



Figure A3a. 72 h *Y. pestis* culture exhibiting a “fried egg” appearance.



Figure A3b. 48 h *Y. pestis* culture with characteristic “hammered copper” morphology.

H. Biochemical reactions/tests

1. **Procedure:** Use established laboratory procedures for catalase, oxidase, and urease tests.
2. **Interpretation:** Follow established laboratory practice.
3. **Additional notes:** Commercial biochemical identification systems are not recommended at this stage.

I. Interpretation and reporting (Fig. A4)

1. **Suspected criteria:** Any isolate, from the respiratory tract, blood or lymph node, containing the major characteristics noted below should be suspected as *Y. pestis*.
Warning: Refer to Section J. Limitations.
 - a. Bipolar staining rod (Wright-Giemsa) on direct smear
 - b. Pinpoint colony at 24 h on SBA
 - c. Non-lactose fermenter, may not be visible on MAC or EMB at 24 h
 - d. Oxidase and urease negative
 - e. Catalase positive
 - f. Growth often better at 28°C
2. **Reporting/appropriate action**
 - a. Level A laboratories should consult with state public health laboratory director (or designate) prior to or concurrent with testing if *Y. pestis* is suspected by the physician (Fig. A4).
 - b. Immediately notify state public health laboratory director (or designate) and state public health department epidemiologist/health officer if *Y. pestis* cannot be ruled out and a bioterrorist event is suspected. The state public health laboratory/state public health department will notify local FBI agents as appropriate.
 - c. Immediately notify physician/infection control according to internal policies if *Y. pestis* cannot be ruled out.
 - d. Preserve original specimens pursuant to a potential criminal investigation and possible transfer to an appropriate LRN laboratory. The FBI and state public health laboratory/state public health department will coordinate the transfer of isolates/specimens to a higher-level LRN laboratory as appropriate. Start chain of custody documentation if appropriate.
 - e. Obtain guidance from the state public health laboratory as appropriate (e.g., requests from local law enforcement or other local government officials).
 - f. If *Y. pestis* is ruled out, proceed with efforts to identify using established procedures.

H. Limitations

1. *Y. pestis* will grow on general nutrient-rich media, but its growth rate is slower than that of most other bacteria; therefore, its presence may be masked by organisms that replicate faster.
2. Bipolar staining of cells is not an exclusive feature limited to *Y. pestis*. *Yersinia* spp., enteric bacteria, and other gram-negative organisms, particularly *Pasteurella* spp., can exhibit the same staining characteristic.
3. Characteristic clumped growth in unshaken broth culture is not an exclusive feature of *Y. pestis*. Some *Y. pseudotuberculosis* and *Streptococcus pneumoniae* can exhibit the same growth features.
4. Some of the automated identification systems do not identify *Y. pestis* adequately. *Y. pestis* have been falsely identified as *Y. pseudotuberculosis*, *Shigella*, H₂S-negative *Salmonella*, or *Acinetobacter* (Wilmoth *et al*, 1996). *Y. pestis* is alkaline slant/acid butt in triple sugar iron. In most conventional biochemical or commercial identification systems, the organism appears relatively inert, making further biochemical testing of little value.

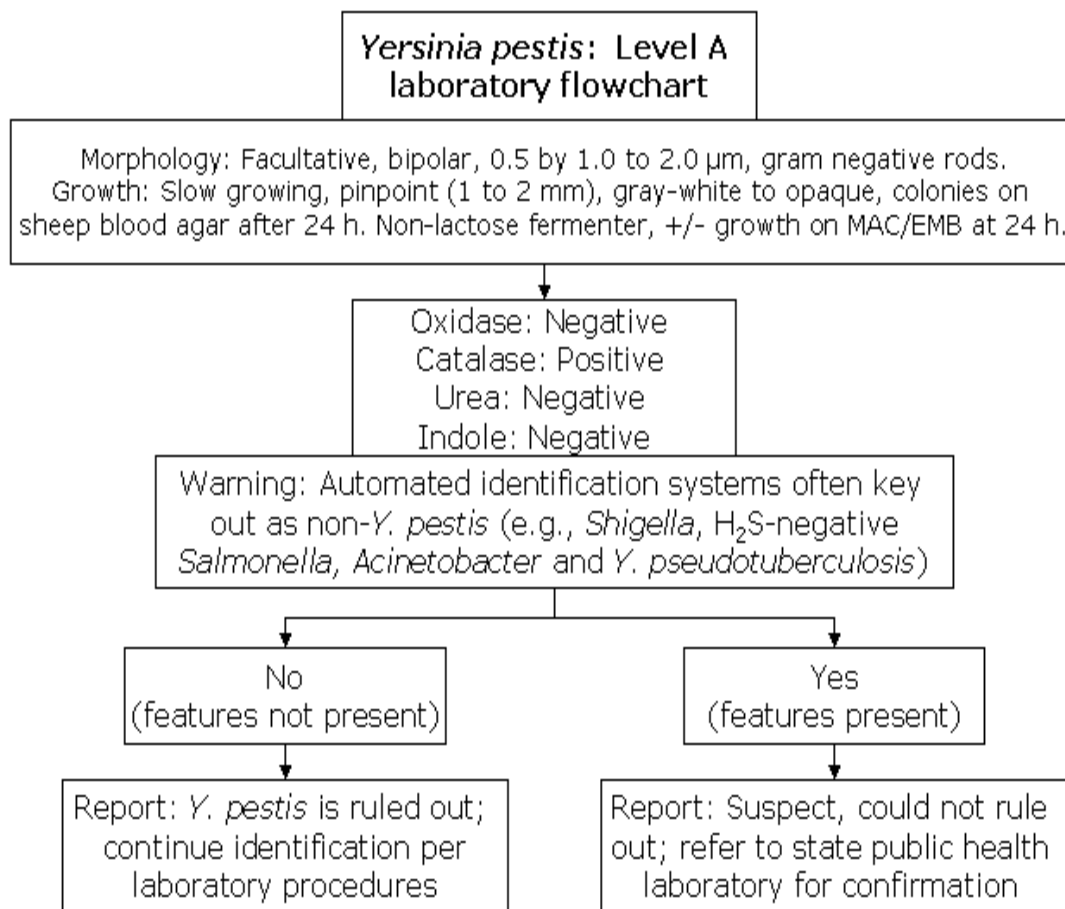


Figure A4. Level A flowchart for *Y. pestis*

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IV. Appendix: Change record

- 1. 22 Apr. 2002**, (ype.la.cp.042202), revised ASM credit information
- 2. 13 Dec. 2001**, (ype.la.cp.121301)
 - a. Section II. I. 2. c. was revised, added the word “out” to “. . . ruled out”.
 - b. Change record added.
- 3. 10 Dec. 2001**, (ype.la.cp.121001): Level A procedure loaded to ASM site.